Abstract

Purpose: To investigate longitudinal changes of subbasal nerve plexus (SNP) morphology and its relationship with conventional measures of neuropathy in individuals with diabetes.

Methods: A cohort of 147 individuals with type 1 diabetes and 60 age-balanced controls underwent detailed assessment of clinical and metabolic factors, neurologic deficits, quantitative sensory testing, nerve conduction studies and corneal confocal microscopy at baseline and four subsequent annual visits. The SNP parameters included corneal nerve fiber density (CNFD), branch density (CNBD) and fiber length (CNFL) and were quantified using a fully-automated algorithm. Linear mixed models were fitted to examine the changes in corneal nerve parameters over time.

Results: At baseline, 27% of the participants had mild diabetic neuropathy. All SNP parameters were significantly lower in the neuropathy group compared to controls ($P<0.05$). Overall, 89% of participants examined at baseline also completed the final visit. There was no clinically significant change to health and metabolic parameters and neuropathy measures from baseline to the final visit. Linear mixed model revealed a significant linear decline of CNFD (annual change rate, -0.9 nerve/mm^2, $P=0.01$) in the neuropathy group compared to controls, which was associated with age ($\beta=-0.06$, $P=0.04$) and duration of diabetes ($\beta=-0.08$, $P=0.03$). In the neuropathy group, absolute changes of CNBD and CNFL showed moderate correlations with peroneal conduction velocity and cold sensation threshold, respectively (r_s, 0.38 and 0.40, $P<0.05$).

Conclusion: This study demonstrates dynamic small fiber damage at the SNP, thus providing justification for our ongoing efforts to establish corneal nerve morphology as an appropriate adjunct to conventional measures of DPN.

Introduction

Diabetic neuropathy is a substantial and burdensome complication of diabetes, affecting up to 50% of these individuals.[1] Diabetic peripheral neuropathy (DPN), which is the most common form of neuropathy, manifests as a distal, symmetric polyneuropathy that begins in the lower extremities and may progress proximally.[2] DPN leads to morbidity in diabetic patients in the form of painful neuropathy and foot ulceration with consequent lower limb amputation.[3] It accounts for reduced quality of life and imposes a significant economic burden that affects both individuals and society.[4, 5]

Several established tests are commonly used for screening, detection and assessment of DPN and to monitor its progression. The majority of these tests examine neuronal function; however, direct observation of nerve structure is also possible. Neurologic symptoms and signs, quantitative sensory tests (QST) and nerve conduction studies (NCS) are the most commonly used tests for DPN.[6] Indeed symptoms, neurological deficits and NCS constitute the basis on which diabetic neuropathy is diagnosed. QST provide quantitative measures of sensation; however, these tests require cooperation and concentration of the examinee and they may also be affected by anthropometric variables.[7] Whilst recent studies have shown that the proficiency of QST assessment is adequate,[8] the reproducibility of symptoms and signs[9] and NCS[10] has been shown to be limited. Furthermore studies in patients with impaired glucose tolerance (IGT)[11] and recently diagnosed type 2 diabetes[12] show a marked small fiber neuropathy accompanying large fiber dysfunction.

Quantification of nerve pathology is possible through direct morphometric examination of nerves including sural nerve biopsy[13] and skin biopsy.[14] However, these techniques are invasive, require expertise for quantification and cannot be repeated from the same site for longitudinal studies. Accurate detection and estimation of progression are needed, especially to test putative treatments, which may alleviate the condition, and/or prevent or delay the development of sequelae. As reviewed in more detail elsewhere,[15, 16] based on the pathogenesis of DPN, several potential therapeutic approaches have been developed targeting these mechanisms; however, apart from glucose control and pain management, currently there is no approved treatment for DPN.[15, 17]

Lack of a sensitive, accurate and reliable clinical endpoint has been one of the obstacles in mounting treatment trials for DPN.[18] Growing evidence supports a prominent association between corneal subbasal nerve plexus (SNP) morphology measured with corneal confocal microscopy (CCM) and DPN. CCM as a quick, non-invasive and reiterative technique has a demonstrated capacity to detect early small nerve fiber damage in diabetic patients,[19] and diagnose[20-22] and classify severity of DPN.[23, 24] Conventional measures of neuropathy and corneal nerve parameters are also related.[21, 23, 25] Furthermore, the demonstration of early corneal nerve regeneration following simultaneous pancreas and kidney transplantation[26] and optimised glycemic and lipid control in an observational study[27] suggests that CCM may well fulfil some of the criteria for a surrogate end point for diabetic neuropathy.

To our knowledge, no study has been conducted to date concerning the natural course of the SNP structure over time in diabetic patients. Therefore in this study, we sought to investigate the natural history of the SNP morphology in type 1 diabetic individuals without and with mild neuropathy. Furthermore, the longitudinal relationship between changes in corneal nerve structure and established measures of neuropathy in individuals with diabetes was also addressed.

Methods

Study Design and Participants

This prospective, observational, longitudinal study was conducted following approval from Queensland University of Technology, Princess Alexandra Hospital, and Mater Hospital research ethics committees as a part of the LANDMark study[28] in Brisbane, Australia. Prior to their enrolment, written informed consent was obtained from all participants and the research adhered to the tenets of the Declaration of Helsinki. Based upon the inclusion/exclusion criteria, 147 type 1 diabetic participants were recruited from Diabetes and Endocrinology Research Centre at Princess Alexandra and Mater hospitals and the general population in Brisbane. Sixty healthy participants, without peripheral neuropathy and/or diabetes were also recruited as controls. All participants were assessed at baseline and assessments continued for four annual subsequent visits (five time-points in total and approximately 960 case visits). Participants were excluded in this study for any of the

following: history of ocular trauma or surgery, ocular disease or systemic disease with potential corneal effect, and systemic disease (other than diabetes). Other causes of neuropathy were excluded. Diabetic participants with moderate and severe neuropathy were also excluded. All participants underwent neurologic and medical evaluation as well as ocular screening (visual acuity, slit lamp examination and intraocular pressure) and CCM, which were repeated annually.

For the definition of DPN, we followed accepted criteria[29] that rely on the presence of abnormal electrophysiological finding, based on age-matched controls at the site, in addition to clinical signs and/or symptoms, which was defined as one or more of the followings: (i) neuropathy disability score (NDS) ≥ 3 of 10,[30] or (ii) diabetic neuropathy symptom score (DNS) ≥ 1 of 4.[31] The methods used during this study to assess neuropathy and clinical and metabolic factors have been reported in detail elsewhere[28] and will be described only briefly here.

Assessment of Neuropathy

Neuropathy signs and symptoms: The neuropathy disability score (NDS), which is a scale of 0 to 10, was employed to assess neurological deficits. This measure included assessment of vibration, pin-prick and temperature perception as well as presence or absence of ankle reflexes in both lower limbs. Diabetic neuropathy symptom score (DNS), a scale of 0 to 4, was used to assess symptoms of neuropathy.

Quantitative sensory tests (QST): QST comprised of vibration perception, measured on the plantar surface of the big toe, and thermal (warm and cold) sensation which was assessed on the dorsal surface of the foot on the hand dominant side.

Nerve conduction studies (NCS): Peroneal motor nerve conduction velocity (ankle to fibula head), amplitude (ankle to extensor digitorum brevis) and F wave latency were determined on the hand dominant side of the participants.

General Health and Metabolic Assessment

At each visit, all participants underwent assessment of height, weight, BMI, blood pressure, HbA_{1c} and lipid profile.

Corneal Confocal Microscopy and Image Analysis

CCM was carried out using the Rostock Cornea Module in combination with a HRT 3 confocal microscope (Heidelberg Engineering, Heidelberg, Germany). Eight images of the SNP, showing in focus nerves and not overlapping more than 20%,[32] were acquired from the centre of cornea on the hand-dominant side using manual focusing and section mode. Automatic segmentation and quantification of the SNP parameters including corneal nerve fiber density (CNFD), branch density (CNBD) and fiber length (CNFL) was performed using ACCMetrics,[33] which is a fully automated analytical system. The SNP parameters for each participant were the average value obtained from the eight captured images and expressed in the unit of number/mm^2 for CNFD and CNBD, and mm/mm^2 for CNFL.

Intra- and Interobserver Repeatability of the SNP Parameters

In order to ascertain the repeatability of the SNP parameters from one time to another, test-retest was carried out by performing CCM examination and automated image analysis for 16 participants including 10 with diabetes and six healthy controls by a single operator as described above. Each participant was examined twice, on the same day of examination, at least 30 minutes apart. No significant differences were found between test and retest measurements for CNFD, CNBD and CNFL ($P = 0.59$, $P = 0.88$ and $P = 0.94$, respectively). The intraclass correlation coefficients (ICC) and coefficient of repeatability (CoR) were 0.81 and 0.08 for CNFD, 0.84 and 0.20 for CNBD, and 0.90 and 0.03 for CNFL, respectively.

To assess the inter-observer reproducibility of the SNP parameters, 11 participants (six with diabetes and five healthy controls) underwent CCM examinations twice by two experienced operators on the same day of examination. The differences of the SNP parameters were not statistically significant for two observers (CNFD, $P = 0.29$; CNBD, $P = 0.22$ and CNFL, $P = 0.21$). The estimated ICC and CoR were 0.87 and 0.10 for CNFD, 0.93 and 0.23 for CNBD, and 0.94 and 0.04 for CNFL, respectively. Overall, CNFL and CNFD achieved the highest values for repeatability and reproducibility, whereas CNBD showed an acceptable consistency within- and between observers.

Statistical Analysis

Normality of the data was examined using the Kolmogorov-Smirnov test and the appropriate test was applied for analysis. Data are presented as mean ± standard deviation (SD) or median and interquartile range (IQR). Four sets of analyses were conducted. First, the demographic and clinical characteristics variables were compared between control and diabetic groups as well as between baseline and final visit. Second, using Toronto criteria, participants with diabetes were stratified into those without DPN (DPN-ve) and with DPN (DPN+ve). Corneal nerve parameters and established neuropathy measures were compared between control, DPN-ve and DPN+ve. For the purpose of the two aforementioned analyses, parametric data were analyzed using the independent samples t test, paired t test, one-way ANOVA and Scheffe post hoc test (pairwise comparison). Nonparametric data were analyzed using the chi square test, Kruskal Wallis test and Mann-Whitney U test.

Thirdly, a linear mixed model was employed to examine changes over time in the SNP parameters and whether the changes were different in the DPN-ve and DPN+ve groups compared with controls. Since change in the SNP parameters (i.e. CNFD, CNBD and CNFL) over time was one of the main parameters of interest of the current study, they were individually considered as response variables and time was added to the model to test the linear effect of time on the response variables. The first model contained CNFD as the response variable, group (i.e. controls, DPN-ve and DPN+ve), time and time*group interaction as primary fixed effects of interest and Type III sum of squares was selected. Group was included as a time-invariant predictor variable to explore any group differences over time.

The association between the initial CNFD parameter and the change in this parameter was estimated by calculating the covariance matrix. Here, the 'variance components' option was chosen and also the restricted maximum likelihood estimates for parameters was used. The process of the aforementioned model was repeated for CNBD and CNFL. Depending on whether the time*group interaction was statistically significant or not, a second set of fixed effects – namely gender, age at enrolment, duration of diabetes, HbA_{1c}, lipid profile, blood pressure, BMI, alcohol and tobacco consumption – were included and their effects were

examined. A stepwise elimination of the variables with non-statistically significant P-values was also applied.

The relation between risk factors and the changes of SNP parameters in diabetic individuals, regardless of their neuropathy status, was analyzed with the latter model where all relevant risk factors were included. Control participants were excluded and group, as factor, was also removed from the model.

Finally, to explore the relationship between changes in corneal nerve parameters and functional measures of neuropathy, absolute change in all parameters was calculated (Δparameter = parameter value at final visit – parameter value at baseline). Bivariate correlations between absolute change of corneal nerve parameters and neuropathy measures were estimated using Pearson r and Spearman's rho correlation coefficients, where appropriate.

IBM SPSS 21 was used for all statistical tests and a two-tailed α=0.05 level of significance was considered for all analyses.

Results

Table 1 shows the clinical characteristics and demographic data of participants with diabetes and controls at baseline and final visit. Approximately 95% of the entering participants were Caucasians of European decent. There was no significant difference between the mean age of participants with diabetes and controls (P = 0.11). There were no statistically significant differences between diabetes and control groups with respect to HDL, triglycerides, diastolic BP, BMI and number of cigarettes smoked per day (P > 0.25). Compared to controls, individuals with diabetes had a higher HbA_{1c} (% NGSP) (P < 0.001) and systolic BP (P = 0.03) and lower total cholesterol (P < 0.001), LDL (P < 0.001) and alcohol consumption (P = 0.001).

The number of participants attending annual visits is depicted graphically in Figure 1. Altogether, 184 participants (89% of the baseline participants) completed the final visit. Personal decision was the main reason for withdrawal (13 participants) followed by poor

health (6 participants). Four participants were also lost to follow up during the study period. The median follow up duration was 3.7 years (range, 3.4 – 4.3) for the cohort.

As can be seen from Table 1, at final visit HbA_{1c} showed a clinically insignificant decrease in controls (mean difference 0.2%, P < 0.001), while it remained the same in participants with diabetes (P = 0.65). Lipid profile, blood pressure, height and alcohol consumption did not differ at final visit compared to baseline visit for both diabetes and control groups (P > 0.05). Whilst BMI showed a statistically significant increase at the final visit in participants with diabetes (P = 0.02), there was no change in controls (P = 0.42). Both control and diabetic participants reported less smoking (number of cigarette/day) at the final visit compared to baseline (P = 0.001).

Comparison of the mean or median change from baseline to final visit in neuropathy measures of individuals with diabetes showed that there were no significant changes in DNS [median 0 (0 – 0) vs. 0 (0 – 0), P = 0.56], cold sensation threshold [median 28.5 (24.8 – 28.5) vs. 28.5 (26.0 vs. 28.5) °C, P = 0.85], vibration threshold [median 6.8 (2.5 – 6.8) vs. 6.6 (2.9 – 6.6) Hz, P = 0.42] and peroneal F wave latency [mean 52.0 ± 5.1 vs. 52.2 ± 7.7 ms, P = 0.85]. NDS [median 1.0 (0.0 – 1.0) vs. 0.0 (0.0 – 0.0), P < 0.01], warm sensation threshold [median 37.6 (34.9 – 37.6) vs. 36.6 (34.8 - 36.6) °C, P < 0.01] and peroneal amplitude [mean 4.6 ± 2.6 vs. 5.0 ± 2.5 mV, P = 0.03] showed slight but significant improvements, whilst peroneal nerve conduction velocity [mean 45.3 ± 6.0 vs. 44.4 ± 5.8 m/s, P = 0.03] was the only measure that declined significantly from baseline to final visit.

Using Toronto criteria, in 147 individuals with type 1 diabetes, 39 (27%) were diagnosed with DPN at baseline. Table 2 delineates the outcomes of the SNP parameters and neuropathy assessment by DPN status. SNP parameters were significantly reduced in DPN-ve and DPN+ve groups compared to controls (P < 0.01). All established neuropathy measures were significantly different between groups. QST, peroneal F wave latency and peroneal amplitude displayed greater deficits in the DPN+ve group compared to DPN-ve and control groups (P < 0.05). Peroneal nerve conduction velocity was significantly lower in both DPN-ve and DPN+ve groups compared to controls and there was also a significant difference between DPN-ve and DPN+ve groups (P < 0.05). NDS and DNS were significantly higher in DPN+ve group compared to control and DPN-ve groups (P < 0.001).

Figure 2 illustrates the 4-year time course for the SNP parameters in the cohort by neuropathy status. The results of the three created basic linear mixed model (LMM) analyses for CNFD, CNBD and CNFL can be found in Table 3. The Type III tests of fixed effects shows overall test of significance for the predictors included in the three basic models (LMM 1-3). There was a significant effect of group for all three models; however the effect of time was not significant for any of them. The Type III F test for the interaction between group and time was only significant in LMM1; therefore no more models were fitted for CNBD and CNFL as response variables.

A second subset of fixed effects was included in LMM1. Upon sequential removal of non-statistically significant fixed effects and considering the lower resultant Akaike's information criteria (AIC) for comparing alternative models,[34] a final model (LMM4) contained the fixed effects of group, time, age, duration of diabetes, HbA_{1c} and the group*time interaction was fitted. Parameter estimates and corresponding standard errors, P-values and 95% confidence intervals are given in Table 4. Group and time did not show a significant effect, while the effects of age at enrolment ($\beta = -0.06$, $P = 0.04$) and duration of diabetes ($\beta = -0.08$, $P = 0.03$) were significant. LMM4 also showed a differential effect of time on the trajectory of CNFD with the slope decreasing by 0.91 nerve/mm^2 for DPN+ve individuals compared to controls (the reference level of the group).

The examination of significant risk factors for corneal neuropathy in diabetic individuals, irrespective of the baseline neuropathy status, showed that CNFD was associated with HbA_{1c} ($\beta = -0.58$, $P = 0.03$) and duration of diabetes ($\beta = -0.08$, $P = 0.03$). CNBD was found to be affected by the duration of diabetes ($\beta = -0.21$, $P = 0.01$) and smoking ($\beta = -0.25$, $P = 0.04$). No statistically significant association was found between CNFL and the included risk factors.

Since peroneal nerve conduction velocity was the only measure that showed a significant worsening in the diabetes group, we sought to compare the trajectories of this parameter between groups utilizing an additional mixed model (LMM5). The above-mentioned basic model was repeated with peroneal nerve conduction velocity as the response variable. There was a significant effect of time ($P < 0.01$) and group ($P < 0.01$), but the group*time

interaction was not significant (P =0.92), indicating that the observed time effect is not different between groups.

In the diabetic group, bivariate correlation revealed a modest association between absolute changes of CNBD and peroneal nerve conduction velocity (Pearson r = 0.23, P = 0.02). In the DPN+ve group, there was a significant correlation between CNBD and peroneal nerve conduction velocity (Pearson r = 0.38, P = 0.05). The absolute change in CNFL was also positively correlated to the cold sensation threshold (Pearson r = 0.40, P = 0.03).

Discussion

In vivo assessment of the SNP morphology using CCM has emerged as a valuable clinical modality to improve our understanding of the relationship between this rich nerve plexus and various ocular and systemic conditions and diseases. As reviewed in more detail elsewhere,[35, 36] morphometric evaluation of the SNP has been used to diagnose, assess and follow up ocular surface conditions including ocular allergy, dry eye, infectious keratitis, and glaucoma and after keratorefractive surgery and contact lens wear. Currently, considerable evidence exists that advocates the utility of CCM for assessment of small nerve fiber pathology induced by systemic and neurological conditions, in particular DPN. This study examined the longitudinal aspect of the utility of CCM to serve as an acceptable measure of DPN in clinical research and practice.

We report data from a cohort of individuals with type 1 diabetes (n = 147) and healthy controls (n = 60) collected from baseline to a median duration of 3.7 years. Although the stability of corneal nerve morphology has been previously demonstrated in a 3-year longitudinal study in healthy individuals,[37] to our knowledge no previous study has examined the dynamic natural course of SNP microstructures in relation to DPN. With reference to the lack of previous investigation concerning the natural history of corneal nerves in diabetes, the present study is a positive response and attempts to fill this research gap.

At the baseline visit, age was matched between participants with diabetes and controls. Diabetic participants showed moderate glycemic control and excellent control of cardiovascular risk factors including the blood pressure and lipid profile in accordance with

the current treatment guidelines.[38] The lower level of total cholesterol and LDL cholesterol in our diabetic patients as compared to controls is attributed to the fact that 35% were receiving lipid-lowering medications.

Comparison of the clinical parameters at baseline and final visit showed that there were no clinically significant changes to health, metabolic and anthropometric measurements, indicating stable glucose control and desirable maintenance of cardiovascular risk factors. Although the Hawthorne effect[39] may have been involved, the finding of lower alcohol consumption in the diabetic patients at baseline which is maintained at follow up reflects good diabetes education. And the significant reduction in tobacco consumption over time in both diabetic patients and control subjects presumably reflects overall population level of education to stop smoking.

Except for peroneal nerve conduction velocity, with a statistically significant but clinically trivial decline (-0.9 m/s), the remaining established measures of neuropathy remained unchanged or improved slightly from baseline to the final visit. However, LMM5 showed that changes in peroneal nerve conduction velocity in DPN+ve and DPN-ve patients did not differ significantly from controls, indicating a similar effect of time for groups. The low rate of change over time in these measures may be attributed to (a) the maintenance of a healthy lifestyle and compliance with medical advice among our diabetic cohort; (b) the inclusion of participants with only mild neuropathy; and (c) the relatively short duration of study. Negligible worsening or no progression of the traditional measures of DPN has also been observed in the placebo arm of a recent interventional study[40] of 227 patients with predominantly type 2 diabetes, but with substantially worse glycemic control at baseline (8.8 $\pm$ 1.9%) and a reduction of 0.67 $\pm$ 1.41% over 4 years. Our findings are further supported by a 3 year longitudinal study of 62 subjects with predominantly type 2 diabetes and good glycemic control (HbA_{1c} 7.23 $\pm$ 1.03%), which interestingly demonstrated stability in a range of neurological examinations, symptom scores, autonomic testing, QST and nerve conduction studies with worsening only in the sural nerve amplitude and the axon-reflex vasodilation test, a measure of small fiber neuropathy.[41]

All three SNP parameters were significantly reduced in diabetic participants without and with neuropathy at the baseline visit. This finding is consistent with other studies, which

also show a depletion of SNP tissue in diabetic patients without and with DPN, reflecting early subclinical small fiber damage.[22-24, 42] Based on the reported association of SNP parameters and DPN severity[23, 24] we hypothesised that participants with diabetes and DPN would demonstrate quicker deterioration of SNP tissue than those without DPN. In order to examine this hypothesis, we built several linear mixed models. Such models afford robust methods of analysing longitudinal data with repeated measurements, in particular when the data is incomplete or unbalanced due to missing data, dropouts or differences in observation time points.[34]

According to the three basic mixed models developed here and regardless of group, there was no significant effect of time for any of the three SNP parameters. A group*time interaction term was not significant for CNBD or CNFL (P = 0.24 and P = 0.20), indicating that the presence or absence of DPN at baseline did not appear to impact CNBD and CNFL changes over time. Mean CNBD (23.7 ± 20.9 vs. 22.7 ± 16.9, no/mm^2) and CNFL (15.0 ± 4.3 vs. 14.4 ± 4.1 mm/mm^2) declined slightly over 4 years in the neuropathy group, but to an extent that is neither clinically nor statistically significant.

However, the Type III F test for the interaction between time and group was statistically significant for CNFD (P = 0.02), suggesting that the relationship of time with CNFD change varies depending on the group. LMM4 (Table 4) demonstrated that whilst CNFD trajectories were not statistically different between DPN-ve and controls, the mean CNFD decreased significantly in the DPN+ve group during follow up, with a loss of approximately 1 nerve/mm^2 per year. This observed CNFD change was best predicted by participant age and duration of diabetes (both P < 0.05). One may anticipate that such a change would be influenced by glycemic control, however, HbA_{1c} did not reach statistical significance (P = 0.10) in LMM4, where CNFD was considered as a dependent variable, possibly because of the relative stability of this factor during the study period. Although the outcome of CNFD decline indicates dynamic structural small nerve fiber damage at the SNP, the relevance of CNFD change in the neuropathy group and the relative stability of CNBD and CNFL are not clear. Disparate changes to these three corneal nerve parameters have also been reported in diabetic individuals after improvement in risk factors for DPN[27] and after simultaneous pancreas and kidney transplantation,[43] suggesting a complex, dynamic and perhaps non-linear relationship between these parameters.

The baseline cross-sectional findings in the present study confirmed that all three SNP parameters were reduced in the neuropathy group compared to controls. The parameter that underwent the most marked reduction over time was CNFD. This suggests that branch damage (thinner branches emanating from major nerves) might represent the primary pathological change in DPN, whereas CNFD (a parameter related to the major nerve trunks) deterioration occurs later. The reduction in CNFD along with a non-significant decline of the other two parameters may also suggest degeneration of major nerve trunks with concomitant regeneration reflected by an increase in the CNBD and CNFL. Therefore, it is conceivable that loss and indeed repair of different SNP parameters may occur at different stages of the disease.

Limited studies are available documenting the link between corneal small nerve fiber change and risk factors of DPN.[21, 27, 44] In the present study, when the data were restricted to include only diabetic individuals and upon removal of the effect of group in the linear mixed models, we found that every one-unit increase of HbA_{1c} was associated with a decrease of ~ 0.6 nerve/mm^2 in CNFD. There also was a negative effect of diabetes duration on CNFD and CNBD. Each 10-year increase of diabetes duration at baseline resulted in 0.8 and 2.0 nerve/mm^2 decline of central corneal CNFD and CNBD, respectively. CNBD was also significantly affected by smoking. Increasing one cigarette per day had a negative effect of 0.25 nerve/mm^2.

These results demonstrate the link between risk factors of DPN and morphologic parameters of corneal nerves. We have no clear explanation why HbA_{1c} has an effect upon CNFD, but not CNBD and CNFL. Nevertheless, this finding is in consistent with the study of Tavakoli et al[27] who reported a significant correlation between changes in HbA_{1c} and CNFD ($r = -0.52$, $P < 0.01$) but not for CNBD and CNFL. In a study of 38 type 1 diabetic patients with and *without* neuropathy, Ishibashi et al[44] reported time-dependent effects of HbA_{1c} on SNP parameters. While nerve beading frequency was positively correlated to the mean HbA_{1c} levels at time of, or up to three months prior to CCM examination, no significant association was found between CNFD and CNFL with HbA_{1c} up to 6 years before CCM examination.

These findings emphasise the importance of including different SNP parameters in future studies, where these parameters are to be used as measures of small nerve fiber damage

and in particular repair. Additionally, in this study, only the central cornea has been investigated. Recent studies have revealed that loss of corneal neve structure in the SNP mainly occurred at the inferior whorl, which is slightly more distal than the central cornea and may therefore be expected to show more marked pathology.[45, 46] Further longitudinal work assessing the inferior whorl as opposed to the central cornea may provide additional insights and ability to discriminate change in relation to DPN.

In previous cross-sectional studies, SNP parameters have been shown to correlate with functional and structural measures of neuropathy.[19, 23, 25] Quattrini et al[19] reported a significant correlation between CNFD versus NDS ($r = -0.30$, $P = 0.03$) and cold sensation threshold ($r = -0.40$, $P < 0.01$). In a subsequent study, moderate correlations were found between NDS and corneal nerve parameters (r_s, -0.48 to -0.58; $P < 0.001$).[23] In a recent study by Sivaskandarajah et al,[25] CNFD, CNBD and CNFL were related to cold sensation threshold (r_s, 0.32 to 0.37; $P \leq 0.01$). In this longitudinal study, we examined the relationship of change in corneal nerve parameter with conventional measures of neuropathy by calculating the absolute change from baseline to final visit for participants with diabetes. We found a modest correlation between CNBD and peroneal conduction velocity (Pearson $r = 0.23$, $P = 0.02$). When the data was restricted to the DPN+ve group, this correlation increased to 0.38. Furthermore, CNFL also correlated to cold sensation threshold ($r = 0.40$, $P = 0.03$), which indicates that SNP parameters do change in a fashion comparable with some traditional measures of neuropathy.

The key strengths of this study are its longitudinal nature, inclusion of a range of traditional neuropathy measures (small and large nerve fiber dysfunction) in a relatively large number of type 1 diabetic participants, the consistency and strict adherence to technical and methodological procedures such as capturing and selection criteria of the SNP images, and employing a fully-automated image analysis algorithm, which is essential to eliminate the variability associated with manual and semi-automated analysis. Thus we used a fully automated image analysis algorithm which has been validated and compared against the manual and semi-automated analysis[33, 42, 47] in individuals with diabetes.

A limitation of this study is that a majority of type 1 participants were enrolled from specialized clinics, where the glycemic and cardiovascular factors were optimally controlled,

which may not represent the typical population with type 1 diabetes. Additionally, four years of study might be insufficient to discern changes over time, particularly in the case of patients with mild neuropathy or the limited number of apparently motivated participants with well-controlled diabetes available in the neuropathy group.

In conclusion, the findings presented herein provide evidence that CCM has the potential to track the structural alterations of the small nerve fibers in DPN. Furthermore, these findings support the notion that quantification of the SNP morphology has a substantial potential to be employed as an appropriate adjunct measure to conventional measures of DPN.

References

1. Dyck PJ, Kratz KM, Karnes JL, et al. The prevalence by staged severity of various types of diabetic neuropathy, retinopathy and nephropathy in a population-based cohort - the Rochester Diabetic Neuropathy Study. *Neurology* 1993;43:817-824.
2. Chin RL, Rubin M. Diabetic Neuropathy. In: Poretsky L (ed), *Principles of Diabetes Mellitus*: Springer US; 2010:357-370.
3. Frykberg RG, Zgonis T, Armstrong DG, et al. Diabetic Foot Disorders: A Clinical Practice Guideline (2006 Revision). *The Journal of Foot and Ankle Surgery* 2006;45:S1-S66.
4. Happich M, John J, Stamenitis S, Clouth J, Polnau D. The quality of life and economic burden of neuropathy in diabetic patients in Germany in 2002 - Results from the diabetic microvascular complications (DIMICO) study. *Diabetes Res Clin Pract* 2008;81:223-230.
5. Van Acker K, Bouhassira D, De Bacquer D, et al. Prevalence and impact on quality of life of peripheral neuropathy with or without neuropathic pain in type 1 and type 2 diabetic patients attending hospital outpatients clinics. *Diabetes Metab* 2009;35:206-213.
6. Dyck PJ, Overland CJ, Low PA, et al. Signs and Symptoms vs Nerve Conduction Studies to Diagnose Diabetic Sensorimotor Polyneuropathy. *Muscle Nerve* 2010;42:157.
7. Boulton AJM, Malik RA, Arezzo JC, Sosenko JM. Diabetic somatic neuropathies. *Diabetes Care* 2004;27:1458-1486.
8. Dyck PJ, Argyros B, Russell JW, et al. Multicenter trial of the proficiency of smart quantitative sensation tests. *Muscle Nerve* 2014;49:645-653.
9. Dyck PJ, Overland CJ, Low PA, et al. Signs and symptoms versus nerve conduction studies to diagnose diabetic sensorimotor polyneuropathy: Cl vs. NPhys trial. *Muscle Nerve* 2010;42:157-164.
10. Litchy WJ, Albers JW, Wolfe J, et al. Proficiency of nerve conduction using standard methods and reference values (Cl. NPhys Trial 4). *Muscle Nerve* 2014.
11. Asghar O, Petropoulos IN, Alam U, et al. Corneal Confocal Microscopy Detects Neuropathy in Subjects With Impaired Glucose Tolerance. *Diabetes Care* 2014.
12. Ziegler D, Papanas N, Zhivov A, et al. Early detection of nerve fiber loss by corneal confocal microscopy and skin biopsy in recently diagnosed type 2 diabetes. *Diabetes* 2014;63:2454-2463.
13. Malik RA, Tesfaye S, Newrick PG, et al. Sural nerve pathology in diabetic patients with minimal but progressive neuropathy. *Diabetologia* 2005;48:578-585.

14. Lauria G, Lombardi R, Camozzi F, Devigili G. Skin biopsy for the diagnosis of peripheral neuropathy. *Histopathology* 2009;54:273-285.

15. Li C, Bunner AE, Pippin JJ. From animal models to clinical practicality: lessons learned from current translational progress of diabetic peripheral neuropathy. In: Souayah N (ed), *Peripheral Neuropathy - A New Insight into the Mechanism, Evaluation and Management of a Complex Disorder*: InTech; 2013:29-76.

16. Varkonyi T, Putz Z, Keresztes K, et al. Current options and perspectives in the treatment of diabetic neuropathy. *Curr Pharm Des* 2013;19:4981-5007.

17. Callaghan BC, Cheng HT, Stables CL, Smith AL, Feldman EL. Diabetic neuropathy: clinical manifestations and current treatments. *Lancet Neurol* 2012;11:521-534.

18. Malik RA. From the bedside to the bench and back again, with corneal confocal microscopy. *Invest Ophthalmol Vis Sci* 2014;55:1231.

19. Quattrini C, Tavakoli M, Jeziorska M, et al. Surrogate markers of small fiber damage in human diabetic neuropathy. *Diabetes* 2007;56:2148-2154.

20. Malik RA, Kallinikos P, Abbott CA, et al. Corneal confocal microscopy: A non-invasive surrogate of nerve fibre damage and repair in diabetic patients. *Diabetologia* 2003;46:683-688.

21. Edwards K, Pritchard N, Vagenas D, Russell A, Malik RA, Efron N. Utility of corneal confocal microscopy for assessing mild diabetic neuropathy: baseline findings of the LANDMark study. *Clin Exp Optom* 2012;95:348-354.

22. Ahmed A, Bril V, Orszag A, et al. Detection of diabetic sensorimotor polyneuropathy by corneal confocal microscopy in Type 1 diabetes A concurrent validity study. *Diabetes Care* 2012;35:821-828.

23. Tavakoli M, Quattrini C, Abbott C, et al. Corneal confocal microscopy: A novel noninvasive test to diagnose and stratify the severity of human diabetic neuropathy. *Diabetes Care* 2010;33:1792-1797.

24. Petropoulos IN, Alam U, Fadavi H, et al. Corneal nerve loss detected with corneal confocal microscopy is symmetrical and related to the severity of diabetic polyneuropathy. *Diabetes Care* 2013.

25. Sivaskandarajah GA, Halpern EM, Lovblom LE, et al. Structure-function relationship between corneal nerves and conventional small-fiber tests in type 1 diabetes. *Diabetes Care* 2013.

26. Tavakoli M, Mitu-Pretorian M, Petropoulos IN, et al. Corneal confocal microscopy detects early nerve regeneration in diabetic neuropathy after simultaneous pancreas and kidney transplantation. *Diabetes* 2013;62:254-260.

27. Tavakoli M, Kallinikos P, Iqbal A, et al. Corneal confocal microscopy detects improvement in corneal nerve morphology with an improvement in risk factors for diabetic neuropathy. *Diabet Med* 2011;28:1261-1267.

28. Pritchard N, Edwards K, Dehghani C, et al. Longitudinal assessment of neuropathy in type 1 diabetes using novel ophthalmic markers (LANDMark): study design and baseline characteristics. *Diabetes Res Clin Pract* 2014;104:248-256.

29. Tesfaye S, Boulton AJM, Dyck PJ, et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. *Diabetes Care* 2010;33:2285-2293.

30. Young MJ, Boulton AJM, Macleod AF, Williams DRR, Sonksen PH. A multicenter study of the prevalence of diabetic peripheral neuropathy in the United Kingdom hospital clinic population. *Diabetologia* 1993;36:150-154.

31. Meijer J, Smit A, Sonderen E, Groothoff J, Eisma W, Links T. Symptom scoring systems to diagnose distal polyneuropathy in diabetes: the Diabetic Neuropathy Symptom score. *Diabet Med* 2002;19:962-965.

32. Vagenas D, Pritchard N, Edwards K, et al. Optimal image sample size for corneal nerve morphometry. *Optom Vis Sci* 2012;89:812–817.

33. Dabbah MA, Graham J, Petropoulos IN, Tavakoli M, Malik RA. Automatic analysis of diabetic peripheral neuropathy using multi-scale quantitative morphology of nerve fibres in corneal confocal microscopy imaging. *Med Image Anal* 2011;15:738-747.

34. Shek DT, Ma C. Longitudinal data analyses using linear mixed models in SPSS: concepts, procedures and illustrations. *The Scientific World Journal* 2011;11:42-76.

35. Villani E, Mantelli F, Nucci P. In-vivo confocal microscopy of the ocular surface: ocular allergy and dry eye. *Curr Opin Allergy Clin Immunol* 2013;13:569-576.

36. Villani E, Baudouin C, Efron N, et al. In vivo confocal microscopy of the ocular surface: from bench to bedside. *Curr Eye Res* 2013;1-19.

37. Dehghani C, Pritchard N, Edwards K, et al. Morphometric stability of the corneal subbasal nerve plexus in healthy individuals: a 3-year longitudinal study using corneal confocal microscopy. *Invest Ophthalmol Vis Sci* 2014;55:3195-3199.

38. American Diabetes Association. Standards of medical care in diabetes—2014. *Diabetes Care* 2014;37:S14-S80.

39. McCambridge J, Witton J, Elbourne DR. Systematic review of the Hawthorne effect: New concepts are needed to study research participation effects. *J Clin Epidemiol* 2014;67:267-277.

40. Ziegler D, Low PA, Litchy WJ, et al. Efficacy and safety of antioxidant treatment with α-lipoic acid over 4 years in diabetic polyneuropathy: the NATHAN 1 trial. *Diabetes Care* 2011;34:2054-2060.

41. Gibbons CH, Freeman R, Tecilazich F, et al. The evolving natural history of neurophysiologic function in patients with well-controlled diabetes. *J Peripher Nerv Syst* 2013;18:153-161.

42. Petropoulos IN, Alam U, Fadavi H, et al. Rapid automated diagnosis of diabetic peripheral neuropathy with in vivo corneal confocal microscopy. *Invest Ophthalmol Vis Sci* 2014.

43. Mehra S, Tavakoli M, Kallinikos PA, et al. Corneal confocal microscopy detects early nerve regeneration after pancreas transplantation in patients with type 1 diabetes. *Diabetes Care* 2007;30:2608-2612.

44. Ishibashi F, Okino M, Ishibashi M, et al. Corneal nerve fiber pathology in Japanese type 1 diabetic patients and its correlation with antecedent glycemic control and blood pressure. *Journal of Diabetes Investigation* 2012;3:191-198.

45. Davidson EP, Coppey LJ, Kardon RH, Yorek MA. Differences and similarities in development of corneal nerve damage and peripheral neuropathy and in diet-induced obesity and type 2 diabetic rats. *Invest Ophthalmol Vis Sci* 2014;55:1222-1230.

46. Edwards K, Pritchard N, Gosschalk K, et al. Wide-field assessment of the human corneal subbasal nerve plexus in diabetic neuropathy using a novel mapping technique. *Cornea* 2012;31:1078-1082.

47. Dehghani C, Pritchard N, Edwards K, Russell AW, Malik RA, Efron N. Fully automated, semiautomated, and manual morphometric analysis of corneal subbasal nerve plexus in individuals with and without diabetes. *Cornea* 2014;33:696-702.

Tables

Table 1. Demographic and clinical characteristics of the participants at the baseline and final visit. Results are expressed as mean ± SD or counts for categorical variable.

	Baseline		Year 4 follow up		P-value		
Parameter	Control (A)	Diabetes (B)	Control (C)	Diabetes (D)	A vs. B	A vs. C	B vs D
n (male/female)	60 (26/34)	147 (71/76)	51 (22/29)	133 (67/66)	0.52*	0.98*	0.73*
Age (years)	51.0 ± 14.7	47.3 ± 15.4	57.0 ± 13.7	52.0 ± 15.3	0.11†	-	-
Duration of diabetes (years)	0	19.8 ± 14.5	0	24.0 ± 14.8	-	-	-
HbA_{1c} (%)	5.4 ± 0.3	8.1 ± 1.4	5.2 ± 0.5	8.0 ± 1.5	< 0.001‡	< 0.001§	0.65#
Total cholesterol (mmol/L)	5.4 ± 1.2	4.7 ± 0.9	5.5 ± 1.1	4.6 ± 0.9	< 0.001†	0.83§	0.23§
HDL (mmol/L)	1.5 ± 0.4	1.6 ± 0.4	1.4 ± 0.3	1.5 ± 0.4	0.26‡	0.06§	0.78#
LDL (mmol/L)	3.5 ± 1.1	2.7 ± 0.8	3.5 ± 1.1	2.5 ± 0.7	< 0.001†	0.96§	0.07§
Triglycerides (mmol/L)	1.1 ± 0.6	1.1 ± 0.6	1.2 ± 0.5	1.1 ± 0.8	0.43‡	0.27§	0.40#
Systolic BP (mmHg)	116.1 ± 13.6	121.0 ± 16.5	117.1 ± 13.7	118.8 ± 12.1	0.03§	0.88§	0.12§
Diastolic BP (mmHg)	72.8 ± 7.0	72.7 ± 8.6	71.7 ± 8.2	71.2 ± 7.0	0.89†	0.27§	0.13§
BMI (kg/m^2)	26.1 ± 5.2	26.5 ± 4.4	26.8 ± 4.9	26.9 ± 4.7	0.46†	0.42§	0.02§
Alcohol consumption (units/week)	5.0 ± 5.7	1.9 ± 1.8	5.2 ± 6.1	1.8 ± 1.8	0.001‡	0.78#	0.20#
Cigarettes smoked (number/day)	6.7 ± 11.5	5.1 ± 8.0	1.3 ± 5.2	1.3 ± 5.6	0.74‡	< 0.001#	< 0.001#

*Chi square test, †Independent samples test, ‡ Mann-Whitney test, § paired samples t test, #Wilcoxon test

Table 2. Baseline comparison of corneal nerve parameters and neuropathy measures in the study participants according to presence and absence of neuropathy defined by Toronto criteria. Outcomes are presented as mean ± SD.

	DPN status at baseline			
Characteristics	Controls n = 60	DPN-ve n =108	DPN+ve n =39	P Group difference
Corneal nerve parameters				
CNFD (number/mm^2)	22.3 ± 8.0	18.3 ± 7.1	16.3 ± 8.3	< 0.001* Controls vs. DPN-ve, DPN+ve†
CNBD (number/mm^2)	35.1 ± 23.8	24.2 ± 17.4	23.7 ± 20.9	0.003‡ Controls vs. DPN-ve, DPN+ve§
CNFL (mm/mm^2)	18.1 ± 3.7	16.0 ± 3.8	15.0 ± 4.3	< 0.001* Controls vs. DPN-ve, DPN+ve†
Quantitative Sensory Tests				
Cold sensation threshold (°C)	28.4 ± 2.8	27.4 ± 5.1	23.4 ± 7.2	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Warm sensation threshold (°C)	38.0 ± 4.1	37.4 ± 3.8	41.6 ± 3.7	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Vibration threshold (Hz)	7.0 ± 8.1	8.7 ± 10.3	25.7 ± 22.2	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Nerve Conduction Studies				
Peroneal F latency (ms)	49.6 ± 5.2	51.5 ± 4.9	55.7 ± 5.0	< 0.001* Controls vs. DPN+ve† DPN-ve. vs DPN+ve†
Peroneal nerve amplitude (mV)	4.7 ± 2.3	5.2 ± 2.7	2.7 ± 1.8	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Peroneal nerve conduction velocity (m/s)	49.0 ± 5.5	46.7 ± 5.0	39.6 ± 5.9	< 0.001* Controls vs. DPN-ve, DPN+ve† DPN-ve vs. DPN+ve†
Neuropathy disability score (0–10)	0.4 ± 0.9	0.6 ± 0.9	2.2 ± 1.5	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Diabetic neuropathy symptom score (0–4)	0.1 ± 0.3	0.2 ± 0.5	1.1 ± 1.0	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§

DPN-ve, diabetic participant without neuropathy; DPN+ve, diabetic participant with neuropathy
* One way ANOVA test, † Scheffe post hoc test, ‡ Kruskal Wallis test, § Mann-Whitney test

Table 3. Results of Type III tests of fixed effects from the three initial linear mixed models analysis. Dependent variables were CNFD in linear mixed model 1 (LMM1), CNBD in LMM2, and CNFL in LMM3.

	LMM1		LMM2		LMM3	
	F	P	F	P	F	P
Intercept	1420.0	< 0.001	423.2	< 0.001	4254.4	< 0.001
Group	8.2	< 0.001	7.4	0.001	10.9	< 0.001
Time (years)	0.03	0.87	1.8	0.18	0.5	0.49
Group*Time	4.0	0.02	1.4	0.24	1.6	0.20

Table 4. Maximum likelihood of the fixed effect parameters for linear mixed model 4, with CNFD as the continuous response variable.

Parameter	Estimate (95% CI)	Std. Error	P-value
Intercept	27.57 (23.01-32.12)	2.32	< 0.001
Time (years)	0.35 (-0.10-0.80)	0.23	0.13
Group			
DPN+ve	-1.36 (-5.17-2.45)	1.94	0.48
DPN-ve	-1.33 (-4.18-1.52)	1.45	0.36
Controls	0*	0	.
Age at enrolment (years)	-0.06 (-0.12-0.00)	0.03	0.04
Duration of Diabetes (years)	-0.08 (-0.16 to -0.01)	0.04	0.03
HbA_{1C} (%)	-0.41 (-0.89-0.08)	0.25	0.10
Group*Time			
DPN+ve * Time	-0.91 (-1.63 to -0.20)	0.37	0.01
DPN-ve * Time	-0.26 (-0.82-0.31)	0.30	0.37
Controls * Time	0*	0	.
* This parameter is set to zero because it is the reference level of the group.			

Figure Legends

Figure 1. Distribution and number of participants examined at various time points. DPN-ve, diabetic participant without neuropathy; DPN+ve, diabetic participant with neuropathy

Figure 2. Longitudinal course of corneal nerve fiber density (A), branch density (B) and fiber length (C) over time. On each graph, the solid line represents control participants, the dashed line represents diabetic participant without neuropathy and the dotted line represents diabetic participant with neuropathy. Error bars indicate mean ± SEM.

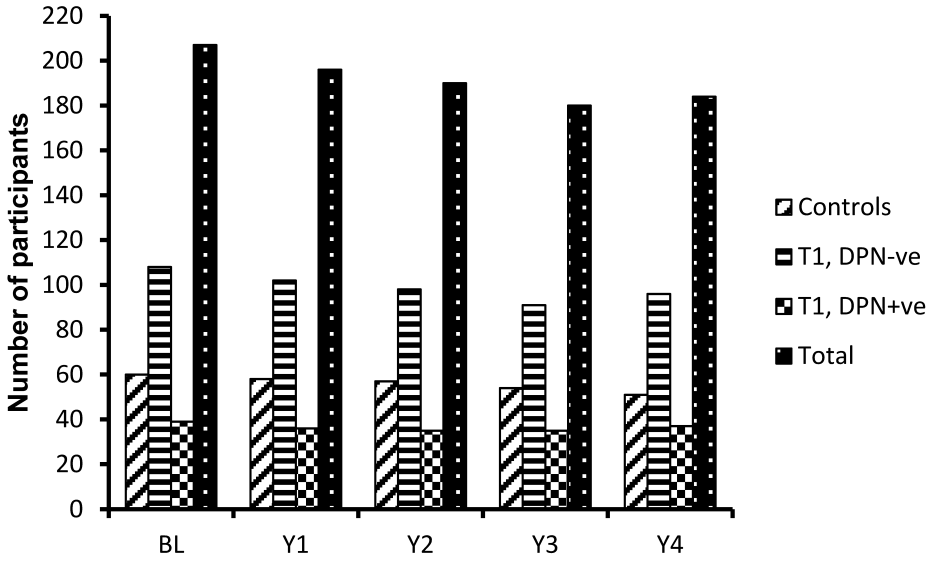
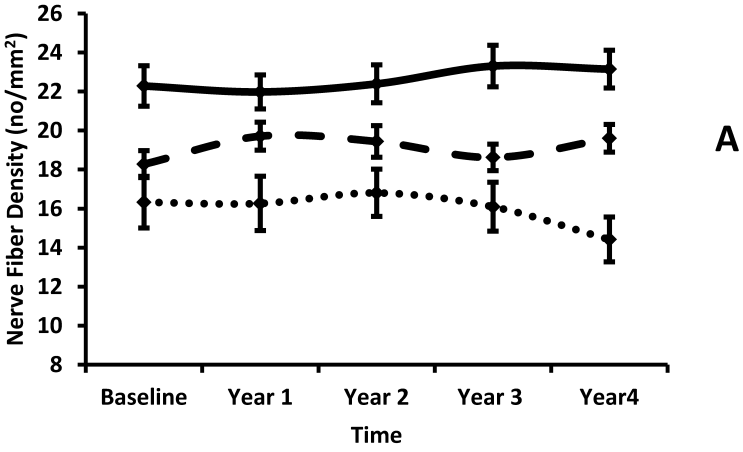
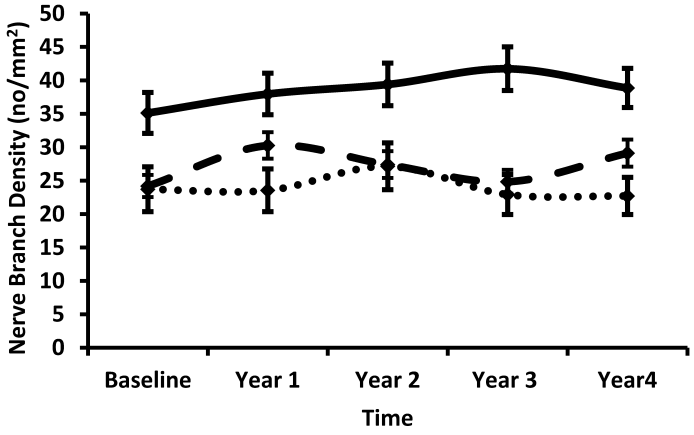

Number of participants
220
200
180
160
140
120
100
80
60
40
20
0
BL
Y1
Y2
Y3
Y4
Controls
T1, DPN-ve
T1, DPN+ve
Total

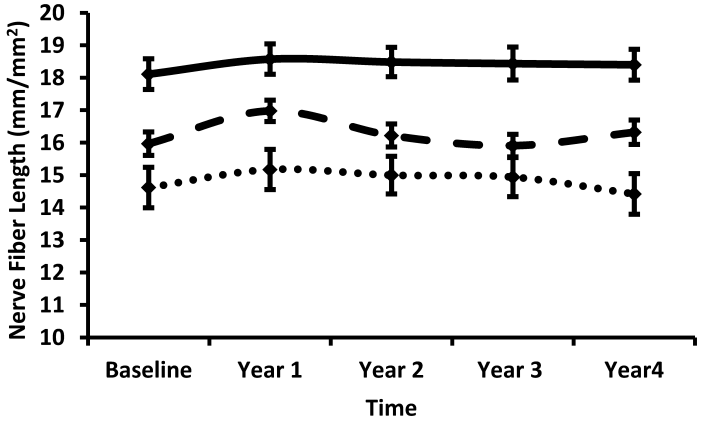
Figure 1


Nerve Fiber Density (no/mm²)
26
24
22
20
18
16
14
12
10
8
Baseline
Year 1
Year 2
Year 3
Year4
Time

A


Nerve Branch Density (no/mm²)
50
45
40
35
30
25
20
15
10
5
0
Baseline
Year 1
Year 2
Year 3
Year4
Time

B


Nerve Fiber Length (mm/mm²)
20
19
18
17
16
15
14
13
12
11
10
Baseline
Year 1
Year 2
Year 3
Year4
Time

C

Figure 2